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3. **Full name, address and postcode of the or of each applicant**
(underline all surnames)

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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation United Kingdom

4. **Title of the invention** Cell Line

5. **Name of your agent** *(if you have one)* D YOUNG & CO

"Address for service" in the United Kingdom to which all correspondence should be sent
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Patents ADP number *(if you have one)* 59006

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Country	Priority application number *(if you know it)*	Date of filing *(day/month/year)*

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D. Young & Co

D YOUNG & CO
Agents for the Applicants

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Cell Line

Field of the Invention

The invention relates to a method for the study of the action of insulin. In particular, the invention relates to a method for studying type-2 diabetes.

Background to the Invention

Diabetes is a major health issue, affecting over 1 million people in the United Kingdom alone. It has been estimated that diabetes and its sequalae account for as much as 5-6% of total NHS spending.

The development of type-2 diabetes is caused by a failure of the pancreas to secrete insulin in sufficient quantities. Type-2 diabetes is usually preceded by a period of insulin resistance. Insulin resistance is caused by impairment of the ability of insulin to properly regulate glucose metabolism. Since muscle is the major site of insulin stimulated glucose disposal, theraputic treatments compensating for insulin resistance in muscle may delay or prevent the onset of diabetes. Therefore, there is a need to fully understand the mechanism of insulin action in muscle.

Considerable effort has been directed towards studying the molecular basis of insulin resistance in human muscle. One approach has been to use *ex vivo* preparations of human muscle (Shepherd *et al.*, 1997: Diabetologia vol 40 pp1172-1177; Shepherd *et al.*, 1997: J. Biol. Chem. vol 272 pp19000-19007). However, studies in intact muscle are technically very demanding due to the possibility of inadvertent stimulation of glucose metabolism by pathways related to muscle contraction. Furthermore, the size of the muscle sample available is often limited. *Ex vivo* muscle preparations are unsuited to experiments which require long term treatments (e.g. testing of drugs) or

manipulations (e.g. transfection studies). Clearly, there are problems associated with prior art *ex vivo* techniques for the study of insulin action.

An alternative prior art approach to the study of insulin action in muscle cells involves the preparation of primary cultures of human myoblasts (Hurel *et al.*, 1996: Biochemical Journal vol 320 pp871-877). Using this approach it has been possible to isolate myoblasts which retain the capability to differentiate into myotubes. However, there are several problems associated with primary cultures of muscle explants. Firstly, the number of cell divisions which the cells will undergo in such cultures is limited and the cells soon senesce, making it impossible to obtain clonal cell lines. Secondly, contamination by fibroblasts is a major problem and affects the interpretation of data obtained from such prior art cultures. Furthermore, growth of these cells is slow (doubling time 4-7 days) and the rate of growth of cells is inversely proportional to the age of the subject from which they were obtained. This slow growth causes practical difficulties associated with the longer periods of cell culture required. In addition, the slow growth can lead to domination of the cell pool by faster growing clones, thus affecting the characteristics of the culture over time. It has been observed that after 10-15 passages these prior art primary muscle cultures lose muscle phenotype. This is a problem.

The present invention seeks to overcome the problems associated with the prior art.

Aspects of the present invention are set out in the claims and are described below.

Summary of the Invention

In a first aspect, the invention relates to a method of studying insulin action in a cell line, wherein the cell line is an immortalised cell line, said method comprising: contacting the cell line with a factor, and determining whether the factor affects insulin action.

The term "immortalised" refers to a cell line which is capable of being cultured through numerous generations without significant senescence or loss of viability.

Preferably, "immortalised" cell lines according to the invention are capable of being cultured indefinitely without encountering a 'crisis' stage.

The "factor" can be any suitable entity for investigation. Without wishing to be bound by theory, it may be a chemical compound or composition, a nucleotide sequence, the expression product of a nucleotide sequence, or a physical factor such as a temperature change and/or an electrical stimulus. The "factor" may be a physiological event, such as exercise or the mimicking of exercise, or it may refer to cellular contraction or signalling events known to be or suspected of being involved in muscle contraction.

The term "insulin action" includes the action of insulin itself or an entity capable of affecting the action of insulin. In a preferred embodiment, the insulin action is associated with Type-2 diabetes.

The term "affects insulin action" is used herein to mean that insulin action is enhanced, increased, augmented, inhibited, reversed, down-regulated or in some way modulated.

The term "affects" is also intended to include mimicking of the effect(s) of insulin, altering the endogenous effect(s) of insulin, or modulating one or more of the effect(s) of insulin. These effect(s) of insulin may be those found in cells or tissues derived from an organism affected by diabetes, such as type-2 diabetes, or may be found in cells or tissues derived from an organism which is not affected by diabetes such as type-2 diabetes.

In another aspect, the invention relates to a method of studying insulin action in a cell line, wherein the cell line is a conditionally immortalised cell line, said method comprising: converting the cell line from an immortalised state to a non-immortalised

state; contacting the cell line when in the non-immortalised state with a factor, and determining whether the factor affects or mimics insulin action.

The term "conditionally immortalised" means a cell that is capable of being in two states: a first state wherein the cell is in an immortalised form; and a second state wherein the cell is in a non-immortalised form, preferably being the same as or similar to the wild type cell. The cells are capable of being converted from one state to the other by changing the environmental conditions of the cell line. By way of example, any one or more of the following parameters may cause said conversion: temperature, pH, pressure, the presence or abscence of specific chemical(s). Typically the cells are prepared by transforming a wild type cell with a reagent which converts the wild type cell to the conditionally immortalised cell.

In one embodiment, the conversion can be achieved, by way of example, with the temperature sensitive tsA58 variant of the large T-antigen. In order to convert the immortalised cell line to the non-immortalised state using the tsA58 large T-antigen as the immortalising agent, the cell line could for example be incubated at elevated temperature, such as 39°C.

In a preferred aspect, the reagent is under the control of an inducible element.

A preferred example of an inducible element is the tamoxifen-responsive estrogen receptor. Preferably this inducible estrogen receptor is fused to an effector domain, for example E1a. Preferably the inducible estrogen receptor is present as an E1a-estrogen receptor chimera. This is discussed in more detail below.

In a preferred aspect, the conditionally immortalised cell is prepared by transfecting (transducing) a wild type cell with a viral vector, preferably a retroviral vector. This topic is discussed at length in (Coffin *et al.*, (ed.s) 1997 "Retroviruses" Cold Spring Harbour Laboratory Press (USA) which is incorporated herein by reference).

In another aspect, the invention relates to a method of studying insulin action in a cell line, wherein the cell line is or is derived from an insulin associated tissue.

An insulin associated tissue is any tissue which is either known or suspected of being involved in some way with insulin. This involvement may be direct, such as tissues which produce insulin, or which control or affect the production of insulin. An insulin associated tissue may be one which responds to insulin in some way, for example by altering its metabolism in response to the prescence or abscence of insulin, or one which has developed resistance to the action of insulin, or resistance to the prescence or abscence of insulin.

In another aspect, the invention relates to a method of studying insulin action in a cell line, wherein the cell line is or is derived from an insulin target tissue.

An "insulin target tissue" is a tissue in which insulin has an effect. This term includes tissues in which insulin would normally have an effect, but which may have developed resistance to the action of insulin. This term also includes tissues in which insulin would not normally have an effect, but may have developed a sensitivity to the action of insulin.

In another aspect, the invention relates to a method of studying insulin action in a cell line, wherein the cell line is or is derived from a tissue selected from muscle, fat or liver. In a preferred embodiment, the invention relates to a method of studying insulin action in a cell line which is, or is derived from, muscle.

In another aspect, the invention relates to a method of studying insulin action in a cell line, wherein the cell line is a transformed cell line.

Transformation may be caused by a number of factors. For example, transformation may be brought about via the expression of a transforming oncogene in the cell(s) of interest. Transformation may be assessed by one or more criteria which are indicative

of the transformed phenotype, for example, a rounded morphology, loss of contact inhibition, growth in soft agar, focus formation, or any other suitable property or marker of transformation known to those skilled in the art.

In another aspect, the invention relates to a method of determining whether an agent can modulate insulin activity in muscle, said method comprising contacting a cell line with an agent, and determining whether the agent modulates insulin activity.

In another aspect, the invention relates to a method of studying insulin action in a conditionally immortalised cell line wherein the cell line is contacted with the agent whilst in the non-immortalised state.

In another aspect, the invention relates to a cell line as described herein, wherein the cell line is for subsequent use for studying Type-2 diabetes.

In another aspect, the invention relates to a vector for use in gene therapy wherein the vector is or comprises a cell line as described herein.

Determining whether a factor affects or mimics insulin refers to the assessment of one or more of the effects of insulin in the prescence and abscence of the factor(s), and deciding whether the factor(s) have influenced one or more of these characteeristic(s) or effect(s). Examples of effects of insulin which might be monitored in order to determine whether or not a factor affects or mimics insulin may include measuring the expression levels of one or more molecules believed to be involved in insulin signalling or glucose metabolism. Other effects which might be monitored include, but are not limited to, measuring the stimulation of one or more of the glucose metabolism and/or insulin-related signalling pathways, monitoring the levels of glycogen synthesis or breakdown, or assessing the activity of enzymes such as glycogen synthase. If any of these characteristics or effects is found to be different in the prescence or abscence of one or more factor(s), then said factor(s) would be

considered to have affected or mimicked insulin action. This aspect is discussed in more detail below.

There exist immortalised muscle cell lines which are currently used for studies of insulin action. However, these prior art cell lines were derived from rodents (e.g. L6 and C2C12). Whilst these cell lines may exhibit myogenic phenotype, the insulin responses in them are poor and do not truly reflect the responses seen in intact human muscle. Such prior art rodent cell lines are therefore of limited value in studying the action of insulin in human muscle. The present invention is therefore advantageous since it provides a means for the study of insulin action in human muscle cells.

Detailed Description of the Invention

Cell lines for use in the present invention may be generated in a variety of ways. For example, it is disclosed herein that human myoblast cultures may be conditionally immortalised using at least two different approaches; firstly, using the temperature sensitive form of the large T-antigen and secondly, using the E1a-estrogen receptor chimera. These techniques are described in more detail below.

When expressed in cells, the temperature sensitive tsA58 mutant of the SV40 large T-antigen may result in immortalisation, so long as the cells are grown at a permissive temperature (such as 33°C). Once the temperature is raised to greater than 37°C (for example 39°C) then the tsA58 is functionally inactivated and the cell growth characteristics revert to being similar to those of the parental cells. The tsA58 large T-antigen may be introduced into primary cells by any suitable means. Advantageously, it may be introduced using amphotropic retroviruses which provide high transfection efficiency combined with stable integration into chromosomal DNA (Stamps *et* al., 1994 Int. J. Cancer vol 57 pp 865-874). After the tsA58 is introduced into the cells, the immortalised cells may be maintained at 33°C in appropriate media.

In another embodiment, cells may be advantageously immortalised using a conditionally active form of the E1a immortalising oncogene. This oncogene is a chimera comprising E1a fused with a Gly525Arg mutant of the C-terminus (amino acids 281-599) of the estrogen receptor. This mutant will not bind estradiol but will bind 4-hydroxy tamoxifen. The E1a chimera is active in the presence of 4-hydroxy tamoxifen, but removal of this drug from the growth medium allows binding of hsp-90 to the chimera which results in the inactivation of the E1a, thus allowing the cell to return to a non transformed phenotype.

There are numerous advantages to using the conditional immortalisation approach which include, among others; (i) all immortalised cells are conferred with the same growth advantage so the characteristics of the cultures remain stable (ii) expansion of cell cultures is much more rapid thus expediting experimental processes such as clonal selection (iii) the immortalization/transformation is reversible, allowing return to a normal muscle phenotype and (iv) cells do not have to undergo chromosomal rearrangement to gain growth advantage and they are far more likely to retain a normal karyotype than immortal cell lines which may arise spontaneously after a crisis period.

Immortalised human muscle cells as described below may be used in conjunction with existing cell culture models in a method according to the invention for study of the action of insulin, for example for study of the insulin signalling pathways regulating glucose metabolism. Preferably, said methods may be applied to muscle cell lines derived from normal and/or diabetic subjects and/or subjects which have or are thought to have a heritable predisposition or susceptibility to the development of diabetes.

It is envisaged that methods according to the invention may identify one or more parts of the insulin signalling cascade which are deranged in human muscle in the diabetic state. Clearly, the methods of the invention may be applied to the study of such defects, and may be usefully employed in carrying out high throughput screening directed at identifying candidate molecule(s) or factors which in some way alleviate, inhibit, reverse or modulate such defect(s).

It would be advantageous to use a transfection approach in the cell lines or methods of the current invention in order to investigate the effect of various factors or wild-type signalling molecules on insulin signalling. Such molecules or factors may be identified by the methods of the current invention, or may be otherwise identified.

It is envisaged that the methods of the current invention may be applied to the analysis of differences in gene expression between normal and diabetic tissues using gene array and/or proteomic methodologies.

We also anticipate that the cell lines and/or methods of the present invention will be of use in studying other diseases affecting skeletal muscle, for example muscular dystrophy or other muscle disorders.

The present invention will now be described by way of example.

EXAMPLE 1: CREATION AND STUDY OF IMMORTALISED RODENT MUSCLE CELL LINES

Primary cultures of mouse muscle precursor cells are derived from "Immortomice" by mincing the tissue and allowing the viable cells to settle onto collagen coated plates in HamsF12 media.

Immortomice are a transgenic line of mice in which a transgene has been introduced encoding the tsA58 temperature sensitive mutant of the large T-antigen under the control of the H2kb promoter. This means that the tsA58 is not actually expressed in most tissues of the mice but is induced to express in cell lines derived from these mice when γ-interferon is added to the cell medium to activate the H2kb promoter.

Therefore, these cell lines are immortalised on the condition they are grown at the permissive temperature of 33°C in the presence of γ-interferon. To induce

differentiation into fused myotubes the cells are allowed to grow to confluency, then γ-interferon is withdrawn to stop production of the immortalising antigen, and any remaining large T-antigen is inactivated by raising the temperature to 39°C, thus preventing further growth. On withdrawal of serum, the cells fuse into myotubes. Three myogenic cell lines are derived in this way (C10, SF1 and SF2).

At this stage the characteristics of the insulin mediated pathways are compared with other systems used for the study of muscle i.e. intact human skeletal muscle, primary cultures of human muscle and with widely used rodent muscle cell lines which were derived from conventional spontaneous immortalization strategies (e.g. L6 cells).

It is demonstrated that the C10 mouse muscle cells and intact human muscle both express the full range of adapter subunit isoforms of the crucial signal transduction enzyme PI 3-kinase. These isoforms are not expressed in the long term primary (non-immortalised) cell cultures of human muscle nor in the L6 cell line.

The regulation of the PI3-Kinase splice variants by insulin in C10 cells is very similar to that seen in intact human skeletal muscle in that both p85 and p50 show very high levels of recruitment to signalling complexes whereas p55 shows very low levels of recruitment. Similarly, C10 cells express high levels of glycogen synthase, a crucial enzyme in glucose metabolism, whereas levels are much lower in human muscle primary (non-immortalised) cell cultures and the L6 cell line.

Overall, these results demonstrate that the conditional immortalisation of the mouse cells allows many of the important characteristics of intact muscle to be retained whereas these are lost in long term primary cultures of human muscle and are also not found in currently available rodent muscle cell lines such as the L6 cell line.

EXAMPLE 2: CREATION OF IMMORTALISED HUMAN MUSCLE CELL LINES

In order to establish primary human muscle cultures, muscle biopsies from type-2 diabetics, non diabetic first degree relatives of type-2 diabetics and control subjects are obtained, and muscle cells are cultured using methods essentially as described in (Hurel *et al.*, 1996: Biochemical Journal vol 320 pp871-877). Biopsies from first degree relatives of type-2 diabetics are included as it has been shown that these subjects share with their diabetic relatives a predisposition to the development of insulin resistance which strongly suggests a genetic basis for insulin resistance. Similarly, the type-2 diabetics chosen for this study have a first degree relative with type-2 diabetes or insulin resistance in order to increase the likelihood that their diabetes originates from a genetic cause. Six subjects in each group are recruited, all subjects being matched for age, sex and body mass index.

Briefly, this is carried out as taught in (Hurel *et al.*, 1996: Biochemical Journal vol 320 pp871-877), and as described in Example 3.

Primary Cell Culture

Muscle biopsy samples (approx. 0.10-0.25 g) are obtained from the gastrocnemius muscle of subjects. Tissue is rinsed in culture medium (Ham's nutrient culture mixture F10 with 20% foetal calf serum, 1% chick embryo extract, 10000 units/ml penicillin and 2 μg/ml streptomycin). All visible connective tissue and fat is removed using forceps, and the biopsy samples are chopped finely with scissors and transferred to a small sterile flask containing 0.05 unit/ml trypsin and 0.05 mM EDTA in PBS. After stirring gently at 37°C for 15 min, the particulate debris is allowed to settle and the supernatant is removed and centrifuged at 550 *g* for 5 min. Pellets are collected and resuspended in conditioning medium. The cells are then grown to confluency in culture medium in 25cm^2 culture flasks coated with 1% gelatin, and are then transferred to 75cm^2 flasks and ultimately to 6cm^2 wells. Fusion into myotubes and

immunocytochemical staining with anti-desmin and anti-(fibroblast surface protein) antibodies confirms the predominant presence of myoblasts.

Characterisation of Muscle Cell Cultures

Myoblast lines are initially tested for their ability to differentiate into myotubes.

The ability of the cells to fuse into multinucleated myotubes is monitored over several days and is assessed morphologically and/or by staining for the muscle marker desmin and/or assaying creatine kinase activity.

These primary cultures of human myoblasts retain insulin resistant characteristics for several passages/generations. Said characteristics are maintained at the time of transfection with immortalising agents.

These primary cell lines are then immortalised. The cultures are transfected with an amphotropic, replication defective retrovirus containing a neomycin selection marker and either the tsA58-Large T-antigen or the E1a-estrogen receptor chimera.

Untransfected cells are eliminated using G-418 selection.

Contaminating fibroblasts are removed from pooled cells by FACS sorting using antibodies to fibroblast surface protein, and clonal lines are then derived.

The cell lines produced maintain insulin resistant characteristics in culture with the added advantage of not having been subject to the diabetic milieu which can itself contribute to the insulin resistance seen at the cellular level.

EXAMPLE 3: STUDY OF INSULIN ACTION IN IMMORTALISED HUMAN MUSCLE CELL LINES

Human cell lines produced as in Example 2 are used to study the action of insulin.

Comparison of insulin responses of cell lines

Expression levels of molecules involved in insulin signalling and glucose metabolism are studied, and the action of insulin on both metabolic and signalling pathways is studied.

Western blotting is used to determine levels of expression of key molecules involved in insulin signalling and glucose metabolism. This is carried out using commonly available antibodies to molecules such as the Glut-1 and Glut-4 glucose transporters, p70S6-kinase, glycogen synthase, glycogen synthase kinase-3, insulin receptor, IRS-1, IRS-2, protein kinase-B and various isoforms of PI3-kinase.

Insulin stimulation of glucose metabolism and/or signalling pathways in different cell lines is studied. Conditionally immortalised myoblasts, myotubes (both normal and diabetic) and L6 myotubes are serum starved for 3 hours, then stimulated for 20 minutes with low (1 nM), medium (10 nM) or high (100 nM) doses of insulin and glucose uptake is assessed using the standard ^{3}H-2-deoxyglucose uptake method (Shepherd *et al.*, 1995: Biochemical Journal vol 305 pp25-28).

The ability of insulin to stimulate glycogen synthesis is determined by measuring the incorporation of U-^{14}C-glucose into glycogen, and insulin stimulated glycogen synthase activity is determined using activity ratios as described in (Shepherd *et al.*, 1995: Biochemical Journal vol 305 pp25-28).

Formation of insulin stimulated phosphotyrosine signalling complexes is assessed. Cells are stimulated with insulin, and tyrosine phosphorylated polypeptides are immunoprecipitated using antiphosphotyrosine antibodies. Immunoprecipitates are

western blotted with antiphosphotyrosine antibodies, and/or with antibodies to the insulin receptor, IRS-1, IRS-2 or the p85 subunit of PI 3-kinase.

To compare the activation of key downstream protein kinase cascades, stimulated cells are lysed and PKB, p70-S6 kinase and MAP Kinase are immunoprecipitated from stimulated and unstimulated control cells using specific antibodies. Kinase activity in immunoprecipitates is determined using myelin basic protein based peptide substrate (APRTPGGRR) for MAP Kinase assays, the crosstide peptide (GRPRTSSFAEG) for PKB assays and the S6 peptide substrate (RRRLSSLRA) for p70 S6 kinase assays. PI3-kinase activity is assayed in immunoprecipitates as described in (Navé *et al.*, 1996: Biochemical Journal vol 318 pp55-60).

Treatment with Insulin

At 16 h prior to the studies, cells are transferred into serum-free medium. When required, insulin is added at a concentration of 1 μM. For inhibition studies, cells are preincubated with 100 nM wortmannin or 100 nM rapamycin for 15 min, or with 50 μM PD98059 for 1 h, prior to the addition of insulin.

Preparation of Cell Extracts

Following incubation of cells under appropriate conditions (ie. with or without insulin stimulation etc.), the cell monolayers are washed rapidly five times with ice-cold PBS.

Extraction buffer [100 mM Tris/HC1, 100 mM KC1, 2mM EDTA, 25 mM KF, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM Na_3VO_4, 1 mg/ml glycogen, pH 7.3, containing 10 μg/ml pepstatin, 10 μg/ml antipain and 10 μg/ml leupeptin] is added (200 μl per 6 cm^2 plate), and cells are scraped and immediately frozen in liquid N_2.

Prior to analysis, samples are thawed, dispersed by sonication for 60s (Sonibath; Dawe) and then centrifuged at 13000 *g* for 5 min at 4°C. The supernatants are retained and the protein concentration is determined.

Fractionation of Cell Extracts on Mono Q

Following centrifugation at 13000 *g*, the supernatant is passed through a 0.2 μm-pore-size filter and the extract, containing approx. 1 mg of protein, is diluted in 5 ml of buffer A (50 mM sodium glycerophosphate, 1 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, pH 7.4, containing 1 μg/ml each of pepstatin, antipain and leupeptin) and loaded on to an FPLC Mono Q column (Pharmacia) equilibrated in the same buffer. After washing extensively with buffer A, proteins are eluted with a linear salt gradient from 0 to 0.5 M NaC1 in 20 ml of buffer A. Fractions of 0.5 ml are collected, and 10 μl of each fraction is assayed for PKB activity as described below or is subjected to immunoblotting using anti-PKB antibodies.

Immunoprecipitation and Assay of Kinases

Samples (30 μl) of cell extract supernatants containing approx. 5 μg of protein are incubated with antibodies to $p70^{s6K}$ (0.2 μg), $p90^{s6K}$ (0.5 μg) or ERK2 (0.4 μg) in a total volume of 40 μl for 2 h at 0°C. Protein A (2 mg) immobililzed on Sepharose 4B-C1 prepared at 100 mg/ml in extraction buffer is added, and the incubation is continued at 4°C for 1 h with occasional shaking.

The immobilized immune complexes are recovered by centrifugation at 13000 *g*, and washed twice with extraction buffer and then once with buffer A. The pellet is resuspended to 20 μl in buffer A, and the kinases are assayed in a total volume of 40 μl containing 50 mM sodium glycerophosphate, pH 7.4, 50 μM [γ-^{32}P]ATP (approx. 4000 c.p.m./pmol), 10 mM $MgCl_2$, 0.5 mM benzamidine, 0.5 mM dithiothreitol, 0.5mM EGTA, 0.05 mM Na_3VO_4, 2.5 μM PKI, and including either 50 μM s6 kinase substrate peptide for the assay of $p70^{s6K}$ and $p90^{s6K}$, or 0.25 mg/ml myelin basic protein for the assay of ERK2. After a 30 min incubation at 30°C, the radiolabelled peptide product is recovered by centrifugation, and 25 μl of the supernatant is spotted on to Whatman P81 phosphocellulose paper squares. After washing in 175 mM phosphoric acid with four changes, the papers are dried and phosphate incorporation is determined by liquid scintillation counting.

GSK-3 immunoprecipitations are carried out using a mixture of anti-GSK-3α and anti-GSK-3β antibodies pre-absorbed in Pansorbin. Cell extracts are prepared in extraction buffer as described above but with the addition of 100 nM okadaic acid. Aliquots containing approx. 10 μg of protein are diluted to 150 μl by addition of buffer A. Following conjugation with antibody/Pansorbin for 2 h at 4°C, samples are centrifuged at 13000 *g* to recover the immune complex and the supernatant is removed. The pellet is washed once with extraction buffer and twice with buffer A. The pellet is then resuspended to 10 μl in buffer A and GSK-3 is assayed in a total volume of 20 μl containing 25 mM sodium glycerophosphate, pH 7.4, 100 mM NaCl, 25 μM GSK-3 phosphopeptide, 50 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 0.5 mM benzamidine, 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.05 mM Na_3VO_4 and 2.5 mM PK1. After a 30 min incubation at 30°C, the radiolabelled peptide product is recovered and quantified as above. GSK-3 is assayed using substrate peptide as described in Borthwick *et al.*, (1995) Biochem. Biophys. Res. Comm. vol 210 pp738-745.

PKB is immunoprecipitated in an identical manner to that for GSK-3, except that anti-(PKB pleckstrin homology domain) antibodies (1 μl) are used instead of anti-GSK-3 antibodies. PKB activity is assayed against 100 μM Crosstide under the conditions described above.

Assay of GS

Following the indicated treatments, cell extracts are prepared in extraction buffer and GS activity is assayed as incorporation of [^{14}C]glucose from UDP-[U-^{14}C]glucose into glycogen. Assays are performed in the presence of low (0.1 mM) and high (10 mM) concentrations of glucose 6-phosphate to give active and total activities of GS, and the results are expressed as fractional activity.

Glycogen Synthesis

Glycogen synthesis is determined as [^{14}C]glucose incorporation into glycogen. Cells are incubated for 2 h in culture medium containing [U^{14}-C[glucose (5 mM glucose; 1.25 μCi/ml) with or without insulin. The experiment is terminated by removing the medium and rapidly washing the cells five times in ice-cold PBS. Cells are lysed by the addition of 20% (w/v) KOH, which is neutralized after 1 h by the addition of 1 M HCl. The wells are aspirated and the contents boiled for 5 min. After addition of 1 mg/ml glycogen, precipitation is carried out with ethanol at 0°C for 2 h. The samples are centrifuged at 1100 *g* for 10 min, pellets are redissolved in water and radioactivity is determined by liquid scintillation counting.

EXAMPLE 4: USE OF HUMAN CELL LINES IN AN ASSAY

Immortalised human muscle cell lines are produced as in Example 2.

These cell lines are used in an assay system to determine the effect(s) of candidate drugs on insulin action.

Cell lines are cultured, and contacted with the candidate drug(s), and then the insulin responses are assayed in said cells, in order to determine whether the candidate drugs have any effect on insulin action, and therefore on type-2 diabetes.

Insulin responses are assayed by monitoring expression levels of molecules involved in insulin signalling and glucose metabolism, and the action of insulin on both metabolic and signalling pathways is studied.

Western blotting is used to determine levels of expression of key molecules involved in insulin signalling and glucose metabolism. Insulin stimulation of glucose metabolism and/or signalling pathways in cell lines contacted with different candidate drug(s) are studied. Formation of insulin stimulated phosphotyrosine signalling complexes is assessed. These analyses are carried out as in Example 3.

The ability of insulin to stimulate glycogen synthesis is determined by measuring the incorporation of U-^{14}C-glucose into glycogen, and insulin stimulated glycogen synthase activity is determined using activity ratios as described in (Shepherd *et al.*, 1995; Biochemical Journal vol 305 pp25-28).

To compare the activation of key downstream protein kinase cascades, stimulated cells are lysed. The various kinases of interest are then immunoprecipitated from cells which were or were not contacted with candidate drug(s). Kinase activity in immunoprecipitates is determined as in Example 3.

Any candidate molecules shown by this assay system to affect insulin action would be considered good drug candidates for use in therapy of Type-2 diabetes.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in this or related fields are intended to be within the scope of the following claims.

Sequence Listing

SEQ ID NO:1

APRTPGGRR

myelin basic protein based peptide substrate

SEQ ID NO:2

GRPRTSSFAEG

crosstide peptide

SEQ ID NO:3

RRRLSSLRA

S6 peptide substrate

CLAIMS

1. A method of studying insulin action in a cell line, wherein the cell line is an immortalised cell line, said method comprising:

(a) contacting the cell line with a factor; and

(b) determining whether the factor affects or mimics insulin action.

2. A method of studying insulin action in a cell line, wherein the cell line is a conditionally immortalised cell line, said method comprising;

(a) converting the cell line from an immortalised state to a non-immortalised state;

(b) contacting the cell line when in the non-immortalised state with a factor; and

(c) determining whether the factor affects or mimics insulin action.

3. A method of studying insulin action in a cell line according to any preceding claim, wherein the cell line is or is derived from an insulin associated tissue.

4. A method of studying insulin action in a cell line according to any preceding claim, wherein the cell line is or is derived from an insulin target tissue.

5. A method of studying insulin action in a cell line according to any preceding claim, wherein the cell line is or is derived from a tissue selected from muscle, fat, or liver.

6. A method of studying insulin action in a cell line according to any preceding claim, wherein the cell line is a transformed cell line.

7. A method of determining whether an agent can modulate insulin activity in muscle, said method comprising contacting a cell line as defined in any preceding claim with an agent, and determining whether the agent modulates insulin activity.

8. A method according to any of claims 2 to 7 wherein the cell line is contacted with the agent whilst in the non-immortalised state.

9. A cell line as defined in any preceding claim wherein the cell line is for subsequent use for studying Type-2 diabetes.

10. A vector for use in gene therapy wherein the vector is or comprises the cell line according to claim 9.

ABSTRACT

CELL LINE

This invention relates to a method of studying insulin action in a cell line, wherein the cell line is an immortalised cell line. This method comprises contacting the cell line with a factor, and determining whether the factor affects or mimics insulin action.